JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1992, p. 2576–2582
0095-1137/92/102576-07$02.00/0

Vol. 30, No. 10

Detection of *Babesia bigemina*-Infected Carriers by Polymerase Chain Reaction Amplification

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Received 30 December 1991/Accepted 6 June 1992

A *Spe*I-*Ava*I fragment (0.3 kbp) from pBbi16 (a pBR322 derivative containing a 6.3-kbp *Babesia bigemina* DNA insert) was subcloned into the pBluescript phagemid vector and was sequenced by the dideoxy-mediated chain termination method. Two sets of primers were designed for the polymerase chain reaction (PCR) assay. Primer set IA-IB was used to amplify a 278-bp DNA fragment, and primer set IAN-IBN was used to prepare a probe directed to a site within the PCR-amplified target DNA. Digoxigenin-dUTP was incorporated into the probe during the amplification reaction. PCR amplification of target DNA obtained from in vitro-cultured *B. bigemina* and nucleic acid hybridization of amplified product with the nonradioactive DNA probe showed that a 278-bp fragment could be detected when as little as 100 fg of parasite genomic DNA was used in the assay. A fragment of similar size was amplified from genomic DNAs from several *B. bigemina* isolates but not from DNAs from *Babesia bovis*, *Anaplasma marginale*, or six species of bacteria or bovine leukocytes. Similarly, the PCR product could be detected in DNA samples purified from 200 μl of blood with a parasitemia of as low as 1 in 10^8 cells and which contained an estimated 30 *B. bigemina*-infected erythrocytes. By a direct PCR method, *B. bigemina* DNA was amplified from 20 μl of packed erythrocytes with a calculated parasitemia of 1 in 10^9 cells. With the analytical sensitivity level of the PCR-DNA probe assay, six cattle with inapparent, 11-month chronic *B. bigemina* infection were found to be positive. No PCR product was observed in bovine blood samples collected from a splenectomized, *A. marginale*-infected bovine, a 4-year chronic *B. bovis*-infected animal, or 20 uninfected cattle from Missouri which were subjected to amplification. The PCR-DNA probe assay was shown to be sensitive in detecting latently infected cattle. The specificity and high analytical sensitivity of the test provide valuable tools for performing large-scale epidemiological studies.

Babesia bigemina is one of several *Babesia* species known to cause bovine babesiosis. The disease is clinically manifested by anemia, fever, hemoglobinuria, and the presence of parasites in the host erythrocytes (20). Recovery from babesiosis is followed by the apparent elimination of parasites from the peripheral blood. However, subclinical infection may last for several years (14).

The serological techniques used to diagnose bovine babesiosis do not consistently detect carrier animals and do not specifically eliminate cross-reactions between *B. bigemina* and *Babesia bovis*, another important hemoparasite (10). Subclinically infected cattle can be proved to be carriers by subinoculation of blood into susceptible splenectomized calves (14).

The use of specific DNA probes and nucleic acid hybridization to detect *B. bigemina* directly in blood from carrier cattle has several advantages over conventional microscopic, serologic, and subinoculation techniques. Although a radioactively labeled probe derived from cloned segments of genomic *B. bigemina* DNA was determined to be highly sensitive (3), radioactive probes have disadvantages for field use because they require frequent labeling, trained personnel, and appropriate facilities. A highly sensitive, nonradioactive DNA-based test would eliminate such shortcomings. However, a nonradioactively labeled DNA probe (6 kbp) prepared to detect *B. bigemina* DNA lacked analytical sensitivity (7). Since the advent of the polymerase chain reaction (PCR) (22), the number of reports on the application of PCR for the diagnosis of infectious diseases has been steadily increasing (18). Thus, the objective of this study was to assess a PCR-based assay with a nonradioactive DNA probe for detecting low numbers of *B. bigemina*-infected erythrocytes present in carrier cattle.

MATERIALS AND METHODS

Source of parasite DNA. Three *B. bigemina* isolates from Mexico and one each from the United States (Texas), St. Croix, Puerto Rico, and Costa Rica and a *B. bovis* isolate from Mexico were maintained in continuous culture as described previously (17, 26). Cultures were expanded in 75-cm^2 flasks as a 10% erythrocyte suspension in medium containing 40% normal bovine serum, 60% medium 199, and 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer. Cultures were incubated in an atmosphere of 5% O_2–5% CO_2–90% N_2 at 37°C, and the spent medium was exchanged at 24 h. Leukocytes were removed from normal erythrocytes by passing blood through a cellulose column (26). *Anaplasma marginale*-infected blood (isolate from Virginia; 80% parasitemia) was obtained from an experimentally infected, splenectomized calf housed in facilities at the National Veterinary Service Laboratories, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, Iowa. Normal bovine blood was col-

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lected from an adult blood donor housed in the Laboratory Animal Research Center of the University of Missouri, Columbia. Genomic DNA from the various organisms was obtained as described previously (15). Briefly, sedimented erythrocytes were frozen at −20°C and were thawed twice at 37°C before resuspending the lysate in 34 ml of TE buffer (0.1 M Tris-HCl [pH 8.0], 10 mM EDTA). Lysates were spun at 12,100 × *g* for 10 min at 4°C, and the pellets were resuspended in 6 ml of TE buffer containing 1% (vol/vol) sodium dodecyl sulfate and 2 mg of proteinase K (Sigma Chemical, St. Louis, Mo.) per ml. After overnight incubation at 37°C, DNAs were then purified by standard phenol-chloroform extraction and ethanol precipitation (23).

Subcloning, DNA sequencing, and primer design. Plasmids pBbi55 and pBbi63 were constructed by inserting a 300-bp *SpeI-AvaI B. bigemina* DNA fragment from pBbi16, a pBR322 derivative containing a 6.3-kbp *B. bigemina* DNA insert (3), into the phagemid cloning vectors, pBluescript II SK(+) and SK(−), respectively, following standard procedures (23) and those recommended by the supplier (Stratagene Cloning Systems, La Jolla, Calif.). Two clones containing the same fragment of foreign DNA but placed in the opposite direction [pBluescript SK(−) and SK(+)] are needed to sequence the plus strand, SK(+), and the complementary strand, SK(−), in order to get the confirmed double-stranded DNA sequence. Purified single-stranded DNA was obtained from clones pBbi55 and pBbi63, and the 300-bp insert was sequenced by the dideoxy-mediated chain termination sequencing reaction by using multifluorescently tagged primers (2). DNA sequencing was performed at the University of Nebraska (Genotype, Inc., Lincoln, Nebr.). Two sets of oligonucleotide primers were designed from the DNA sequence and synthesized in an Applied Biosystems DNA synthesizer (model 380B) at the University of Missouri DNA Core Facility. By using primers IA (5′-CATCTAATT TCTCTCCATACCCCTCC-3′) and IB (5′-CCTCGGCTTCA ACTCTGATGCCAAAG-3′) in the PCR test, an amplified 278-bp DNA fragment would be obtained, whereas a 170-bp sequence within the 278-bp target would be amplified with primers IAN (5′-CCGACCTGGATAGGCTGTGTGATC-3′) and IBN (5′-CCGACCTGGATAGGCTGTGTGATG-3′), which were used to prepare the probe (see below).

DNA amplification by PCR. For specificity analysis, 100 ng of purified plasmid, *Babesia* spp., *A. marginale*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Pasteurella haemolytica*, *Brucella abortus*, *Moraxella bovis*, or bovine leukocyte DNA was placed in 98.5 μl of reaction mixture buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM [each] deoxynucleoside triphosphate [dNTP], 1 μM [each] primer) and was denatured at 95°C for 5 min. After the addition of 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn., or Promega Corporation, Madison, Wis.), PCR tubes were placed in a TempCycler 50 (Coy, Ann Arbor, Mich.) and the reactions were performed under the following temperature profile: 1 min of template denaturation at 95°C, 1 min of primer annealing at 65°C, and 1.5 min of primer extension at 73°C for a total of 35 cycles, with a final extension at 72°C for 15 min. For the sensitivity analysis, 10-fold dilutions of purified *B. bigemina* DNAs were made, and the DNAs were subjected to PCR amplification as described above. To avoid amplicon contamination during the PCR procedure, individual steps (DNA extraction, PCR mixture preparation, PCR product analysis) were performed in separate rooms, and dedicated pipets and tips were used (11).

In order to amplify *B. bigemina* DNA from infected blood, two procedures were tested. Procedure 1 (7) included digestion of 200-μl aliquots of 10-fold dilutions of packed *B. bigemina*-infected erythrocytes with 200 μl of TE buffer containing 0.5% (vol/vol) Triton X-100 (Bio-Rad Laboratories, Richmond, Calif.) and 400 μg of proteinase K (Sigma) per ml; this was followed by purification of DNA from blood lysates by using a slurry of DNA-binding silica matrix (Geneclean Kit; Bio 101, Inc., San Diego, Calif.). Water eluates (prepared by adding 10 μl of deionized water to the DNA-bound silica matrix and incubating the samples at 56°C for 5 min) were diluted in the reaction mixture buffer, and the PCR assay was carried out as described above. Procedure 2 was based on a recently published technique (24) with minor modifications. Briefly, 20 μl of washed, leukocyte-depleted, packed infected erythrocytes containing various amounts of *Babesia* parasites were placed in lysis buffer (0.015% saponin, 35 mM NaCl, 1 mM EDTA) and vortexed gently. After 15 min of centrifugation at 12,000 × *g* and 4°C, the hemoglobin was removed by aspirating the supernatant with a pipet tip, and the pellet was resuspended in 250 μl of reaction mixture buffer (as described above, but without dNTPs and primers) and centrifuged again. Pellets were directly resuspended in 99.5 μl of reaction mixture buffer containing dNTPs and primers, and the suspension was incubated at 100°C for 10 min in the thermocycler. After a brief centrifugation, 2.5 U of *Taq* polymerase was added and the reactions were performed for 35 cycles by using the temperature profile described above, except that the first cycle included a 2-min denaturation step at 95°C.

Experimental animals. Peripheral blood samples from animals (group A) experimentally infected with *B. bigemina* (eight bovines; samples were collected 10 to 15 days postinoculation [p.i.]), *B. bovis* (1 bovine; 4-year carrier), and *A. marginale* (one splenectomized calf; blood was collected at the time of peak parasitemia, 26 days p.i.). Blood was also collected from 20 uninfected cattle from a ranch in Missouri. Samples were processed as described previously (7), and packed erythrocytes were kept frozen at −20°C until they were used. After two freeze-thaw steps, blood samples were treated as outlined above for PCR procedure 2 (direct PCR). In addition, six *B. bigemina*-infected calves (group B) were monitored by using the PCR assay at various times from the inoculation day until 11 months postinfection. PCR results were compared with those obtained by light microscopy of Giemsa-stained blood smears, serological assay-indirect fluorescent antibody test (9) and complement fixation test (13), in vitro culture parasite isolation (26), and blood subinoculation (blood from two animals only) into a susceptible splenectomized calf (14).

Preparation of PCR-labeled probe. Purified pBbi16, pBbi55, or pBbi63 plasmid DNA served as a template (10 ng) for the synthesis of a probe by PCR and by using oligonucleotides IAN and IBN as primers. The procedure was performed by using the temperature profile described above; it was adapted from a previously reported technique (6) in which dTTP is replaced at about 35% with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, Ind.). Purification of digoxigenin-labeled DNA was carried out by ethanol precipitation of the probe in the presence of 20 μg of glycogen and 0.4 M LiCl. The amount of digoxigenin-labeled probe was approximated by comparison with a labeled control DNA (Genius kit; Boehringer Mannheim) in a direct detection method according to the instructions of the manufacturer. Removal of unincorporated dNTPs and estimation of the amount of labeled DNA probe was conducted as suggested by the supplier.

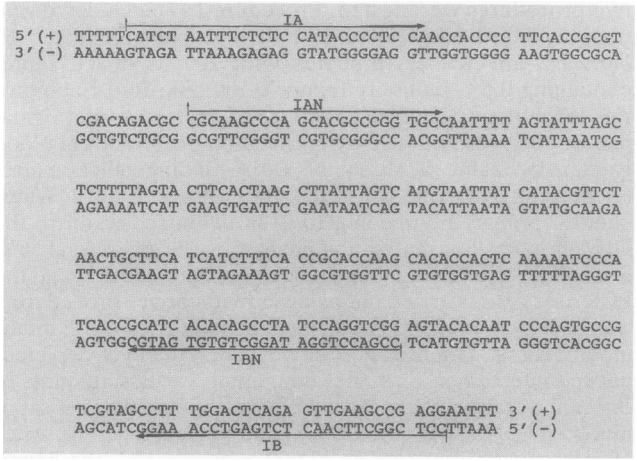

FIG. 1. Double-stranded DNA sequence of the 300-bp *B. bigemina SpeI-AvaI* restriction fragment contained in pBbi55 and pBbi63. The primers that were designed are illustrated by lines; arrows indicate the direction of polymerization.

Analysis of PCR products. Twenty microliters of the amplification products was analyzed by electrophoresis on 1.5% low-melting-point agarose gels (Bethesda Research Laboratories, Gaithersburg, Md.) by using standard Tris-borate-EDTA buffer containing 1 μg of ethidium bromide per ml, and bands were visualized by transillumination with UV light (23). For Southern blot analysis, the DNA in agarose gels was depurinated, denatured, and neutralized as described previously (23). The DNA in the gels was then vacuum transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, N.H.) by using a Vacublot apparatus (American Bionetics, Inc., Hayward, Calif.) as recommended by the manufacturer. For dot blot analysis, 20 μl of the PCR was spotted onto nylon membranes by using a dot blot apparatus (Schleicher & Schuell). DNA was denatured by placing the membranes on filter paper saturated with 0.5 M NaOH–1.5 M NaCl for 15 min at 37°C; this was followed by neutralization with 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl for 15 min at 4°C. Nylon membranes were then hybridized with the nonradioactive probe (20 to 100 ng of hybridization solution per ml) as described in the Boehringer Mannheim applications manual. DNA hybrids on Southern blots were detected with an antidigoxigenin-alkaline phosphatase conjugate; this was followed by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Genius kit), as suggested by the supplier. The DNA hybrids on the dot blots were detected by using a chemiluminescent substrate for alkaline phosphatase (Lumiphos 530; Boehringer Mannheim), this was followed by a 15-min exposure of membranes to X-OMAT film (Eastman Kodak, Rochester, N.Y.).

RESULTS

Several clones carrying the pBluescript vector containing the 300-bp *B. bigemina* DNA insert were isolated (data not shown). pBbi55 and pBbi63 were selected to confirm the DNA sequence. The confirmed double-stranded DNA sequence and the primer orientation are shown in Fig. 1.

By using primer sets IA-IB, IAN-IB, and IAN-IBN, PCR-amplified products of the predicted sizes (278, 223, and 170 bp, respectively) could easily be detected in the agarose gel under UV illumination when 10 ng of plasmid DNA was used as the template (Fig. 2). By using primer set IA-IB in the PCR, amplification of purified parasite DNA showed the expected 278-bp fragment in all the geographically different *B. bigemina* isolates. The amplified product was parasite and species specific, as demonstrated by the lack of DNA amplification in reactions containing *B. bovis*, *A. marginale*, *S. aureus*, *S. typhimurium*, *E. coli*, *P. haemolytica*, *B. abortus*, *M. bovis*, and bovine leukocyte DNA templates (data not shown).

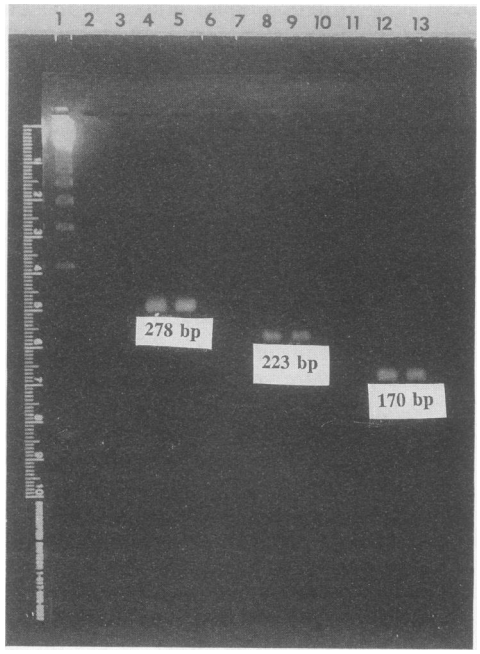

FIG. 2. Analysis of PCR products by gel electrophoresis. Lanes: 1, 123-bp ladder markers (Bethesda Research Laboratories); 2, 6, and 10, empty wells; 3, 7, and 11, no template DNA (negative controls); 4, 8, and 12, pBbi55 template DNA; 5, 9, and 13, pBbi63 template DNA. Lanes 3 to 5 contain PCR products of samples amplified by using the external primer set IA-IB; lanes 7 to 9 contain PCR products of samples amplified with one external (IB) and one nested (IAN) primer; lanes 11 to 13, contain PCR products of samples amplified by using the nested primer set IAN-IBN.

Sensitivity studies showed that the 278-bp fragment could be physically visualized in reactions containing as little as 10 pg of parasite template DNA. The sensitivity detection level could be increased by 2 orders of magnitude by nucleic acid hybridization with the nonradioactive probe in the Southern blot analysis, although the color reaction was not reproduced photographically in the PCR sample containing 100 fg of parasite DNA as the template (Fig. 3). Analysis of PCR products obtained with DNA prepared by procedure 1 revealed bands of the expected sizes in samples containing DNA purified from as little as 30 infected erythrocytes (0.000001% infected erythrocytes in a 200-μl PCV sample containing an estimated count of 3×10^9 erythrocytes) by Southern blot hybridization (data not shown). Analysis of PCR products amplified directly from *B. bigemina*-infected erythrocytes (procedure 2) showed ethidium bromide-stained bands in sample reactions containing from 3×10^6 to 3×10^3 infected cells (1 to 0.0001% infected erythrocytes in a 20-μl PCV sample with an erythrocyte count of 3×10^8 total bovine erythrocytes) by Southern blot probe hybridiza-

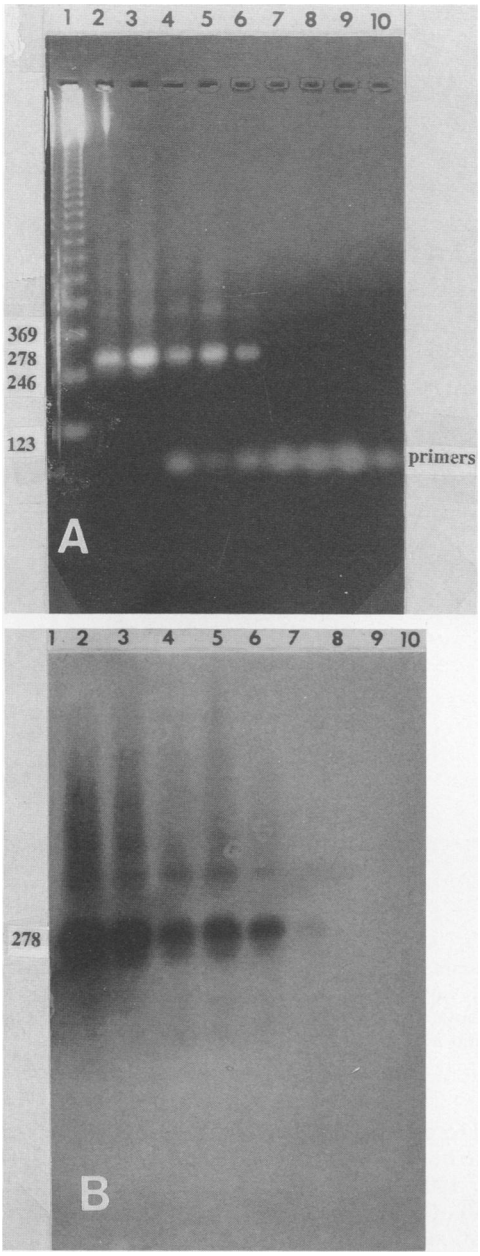

FIG. 3. Detection limits of PCR on *B. bigemina* purified DNA by agarose gel electrophoresis (A) and Southern blot hybridization with digoxigenin-labeled internal probe (B). Tenfold dilutions of *B. bigemina* DNA were subjected to PCR amplification with primer set IA-IB by procedure 2 as outlined in the text. Lanes: 1, 123-bp ladder markers; 2, 100 ng of DNA; 3, 10 ng of DNA; 4, 1 ng of DNA; 5, 100 pg of DNA; 6, 10 pg of DNA; 7, 1 pg of DNA; 8, 100 fg of DNA; 9, 10 fg of DNA; 10, no template DNA (negative control).

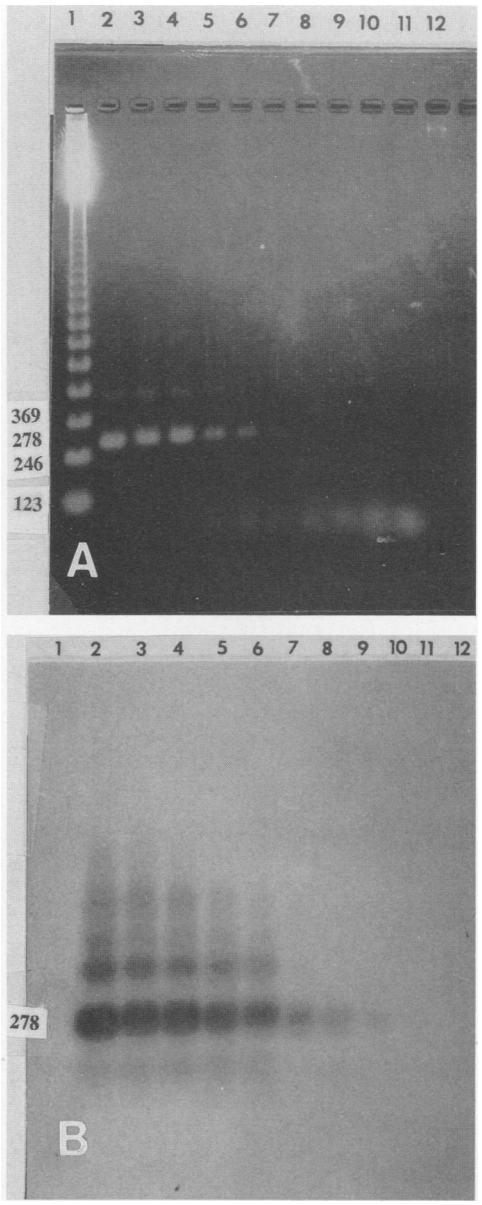

FIG. 4. Detection limits of PCR on *B. bigemina*-infected erythrocytes by agarose gel electrophoresis (A) and Southern blot hybridization with digoxigenin-labeled internal probe (B). Tenfold dilutions of *B. bigemina*-infected erythrocytes were subjected to PCR amplification with primer set IA-IB by procedure 2 as outlined in the text. Lanes: 1, 123-bp ladder markers; 2, 3,000,000; 3, 300,000; 4, 30,000; 5, 3,000; 6, 300; 7, 30; 8, 3; 9, 0.3; 10, 0.03; 11, 0 (all numbers are the amount of infected erythrocytes present in 3×10^8 bovine erythrocytes).

tion (Fig. 4). The level of detection was increased by at least 3 orders of magnitude (0.0000001%) by Southern blot probe hybridization (Fig. 5). It should be noticed that two or three more bands with sizes larger than the predicted 278-bp fragment were also amplified. These bands were more readily detected with the probe, particularly in PCRs containing relatively large amounts of template DNA. These bands were *B. bigemina* specific, however, as evidenced by the absence of such products in PCR tubes containing heterologous template DNA (*Babesia* sp. DNA, *A. marginale* DNA, or bovine leukocyte DNA) (data not shown). Figure 5 shows that all eight calves (group A) experimentally infected with *B. bigemina* were found to be positive by the PCR test. These animals had inapparent clinical infections, and six of them had been diagnosed as positive by light microscopy after only a few infected erythrocytes were found in an exhaustive examination of Giemsa-stained blood smears. Results of PCR-dot blot analysis of blood from the

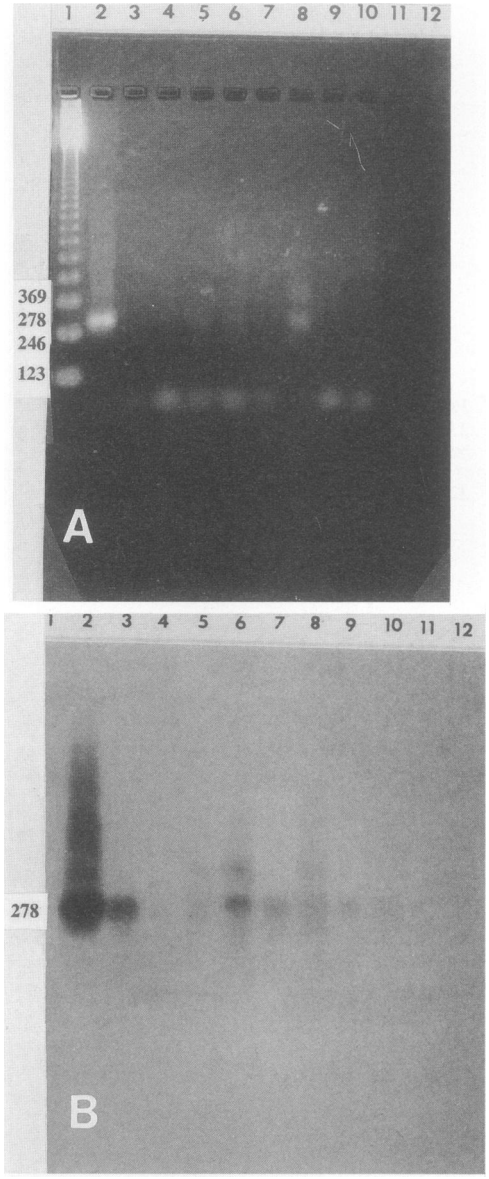

FIG. 5. Analysis of PCR-amplified DNA products of bovine blood from experimentally infected cattle (group A) by agarose gel electrophoresis (A) and Southern blot hybridization with digoxigenin-labeled internal probe (B). Lanes: 1, 123-bp ladder markers; 2, positive control (3,000 *B. bigemina*-infected erythrocytes); 3 to 10, *B. bigemina*-infected cattle (3, animal 182; 4, animal 175; 5, animal 335; 6, animal 100; 7, animal 59; 8, animal 68; 9, animal 1168; 10, animal 1225; 11, animal 433 [*B. bovis*-infected carrier]; 12, *A. marginale*-infected calf).

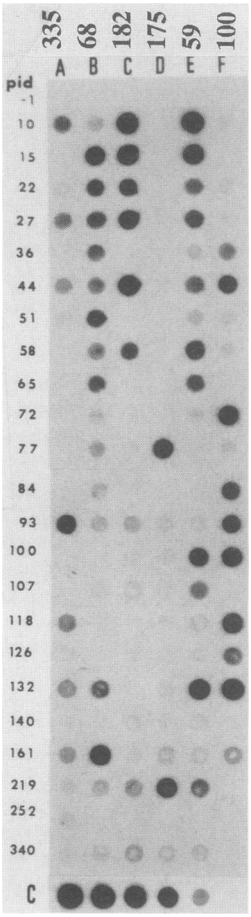

FIG. 6. Analysis of PCR products by dot blot hybridization-Chemiluminescence detection of *B. bigemina*-infected cattle (group B). pid, days postinoculation; animal identification numbers are given above the lanes; c, positive (300,000 to 30 infected erythrocytes) and negative controls.

six infected animals (group B) monitored through 11 months are shown in Fig. 6.

Animals 335 and 175 were found by light microscopy to be *B. bigemina* negative throughout the entire 11-month experiment. The other four calves showed variable light microscopy-positive results from days 10 to 65 (p.i.). Bovine 68 had light microscopy-detectable organisms on days 15, 17, 22, 24, and 51 p.i.; however, the parasitemia (0.054%) could be estimated only on day 17. Animal 182 showed parasitemias of 0.014, 0.028, 0.034, <0.01, and <0.01% on days 10, 13, 15, 17, and 65 p.i., respectively. Only on 2 days p.i. was steer 59 found to be positive, on day 10 (0.26%) and day 51 (<0.01%). Bovine 100 was positive by microscopic examination on days 10 (0.038%), 13, 15, 22, and 44 (<0.01%) p.i. In contrast, all six calves inoculated with *B. bigemina*-infected blood were found to be positive by the PCR-based assay. The first time they were found to be PCR positive was at day 10 p.i., but animal 175 had a weakly positive PCR at day 27 p.i., but it was definitely positive by day 77 p.i. (Fig. 6). The signal intensity detected in dot blots varied between sampling dates and among the infected animals, from negative reactions on some days to strong reactions throughout the study period, indicating the absence or presence of different levels of parasitemias on a given day. Except for bovine 100, all animals were PCR positive at the end of the sampling period (340 days p.i.). The PCR results for the bovine samples analyzed were considered to be accurate because none of the 20 bovine blood samples collected from a *Babesia*-free area was positive by the PCR assay (data not shown). In general, the number of times an animal's sample was positive by the PCR-based assay was always higher than that determined by light microscopy. Thus, for 13 to 21 times (of 23 times tested), the samples were PCR positive in the postinoculation period analyzed. Two animals did not show

peripheral parasites, and on only two to four occasions were samples from the other four calves positive by light microscopy examination.

Serologically, animals 335, 68, 182, and 100 were positive by day 86 p.i. However, all the calves were serologically negative at day 219 p.i. *B. bigemina* parasites were isolated in culture from the blood of animals 335, 68, 182, 175, and 100 at day 140 p.i.; and only animal 335 was parasite culture positive at 161 days p.i. Three more attempts to isolate *B. bigemina* from the animals by in vitro cultivation were unsuccessful at days 219, 252, and 340 p.i. Blood collected from animals 175 and 335 approximately 1 year p.i. was pooled and 500 ml was inoculated into a splenectomized calf to obtain proof of their carrier state. The recipient calf became *B. bigemina* infected 7 days after blood transfer, as evidenced by light microscopy examination of blood smears.

DISCUSSION

Diagnosis of cattle clinically infected with *B. bigemina* is relatively simple on the basis of the manifestation of the disease and the presence of infected erthrocytes in peripheral blood. Accurate diagnosis of subclinically infected cattle is, however, more difficult, since conventional microscopic and serologic techniques lack the reliability and sensitivity features that are required in a diagnostic test (1). This prompted us to develop a highly sensitive, specific, nonradioisotopic PCR-based test to detect parasite DNA directly in blood from chronically infected cattle.

DNA amplification of purified *B. bigemina* DNA from all geographically different parasite isolates resulted in fragments of similar sizes. This result indicates the presence of similar sequences that are conserved among the parasite populations of North and Central America and the Caribbean region. Further analysis with parasite DNA from other regions of the world would confirm the global conservancy of the 278-bp *B. bigemina* sequence. This feature would be highly desirable in a diagnostic test, since a protein-based serologic test (i.e., the slide agglutination test) has been used and has demonstrated the antigenic differences among *B. bigemina* strains (5).

The analytical sensitivity of the PCR-based test reported here (100 fg) was 10,000 times greater than that detected in an unamplified target DNA by using a nonradioactive DNA probe (7) and was at least 100 times more sensitive than a ^{32}P-labeled probe (3). The increased sensitivity of the PCR test against the other configurations was also confirmed when it was estimated as the percentage or number of infected erythrocytes in a blood sample. Thus, parasitemias of 0.01 to 0.001% (3×10^5 to 3×10^6 infected erythrocytes) detected in a 200-μl blood sample with the nonradioactive DNA probe (7) is considered of relatively low sensitivity in an assay compared with the detection limit offered by the PCR-based assay (0.000001%). One of the major drawbacks of the PCR assay conducted by procedure 1 (DNA purification with glass powder) is the tedious and time-consuming protocol required. In addition, an experienced laboratory technician could handle no more than 40 samples at a time. Therefore, the need for a less cumbersome sample preparation procedure, but that, at the same time, maintains the high level of sensitivity of the PCR-based assay, was evident. By using the direct PCR assay, not only the DNA purification step was obviated but the level of detection was increased to 0.0000001% (less than one infected erythrocyte) in a smaller, packed blood cell volume (20 μl). Indeed, this level of sensitivity has previously been reported in *Plasmodium falciparum*-infected human erythrocytes when 20 μl of whole blood was used to provide for template DNA for PCR amplification and probe hybridization (24).

Efficient methods that can be used to amplify DNA directly from whole-blood samples without DNA purification have been described (16). However, use of only 1 or 2 μl of whole-blood samples was recommended because of the potential of the *Taq* polymerase inhibitors (such as hemoglobin) present in blood. As little as 4 μl of whole blood in the PCR vessel totally inhibited the amplification reaction. It appears that the blood lysis and washing steps involved in procedure 2 used in this study eliminated most of the hemoglobin and its inhibition potential.

The PCR-Southern blot configuration definitely proved the specificity and sensitivity of the *B. bigemina* PCR-based assay. However, analysis of a large number of samples by this procedure would be a very expensive and lengthy process. Thus, a PCR-dot blot configuration that could handle up to 100 samples at a time and that could provide results in 2 days was evaluated. Moreover, by using chemiluminescent detection of DNA hybrids, the system proved to be more rapid and sensitive than the colorimetric one in the dot blot format, since the weakly positive hybridization signals were more easily discernible from the background in an exposed X-ray film than was the spot developed in the nylon membrane. With this PCR system, light microscopy and serologically negative cattle were shown to be infected with *B. bigemina*. This was corroborated by doing the subinoculation experiment in which at least one of the blood donors had a circulating parasitemia level high enough to induce infection in the recipient calf. Lack of a sufficient number of recipient calves precluded the demonstration of the carrier state of all animals by blood subinoculation. However, on the basis of the PCR-based assay and in vitro culture isolation results, we feel confident that all cattle remained infected for at least 1 year p.i. The duration of latent *B. bigemina* infection in naturally infected cattle has been reported to last from 11 to 57 months after primary infection (12), from 5 to 22 months (19), or longer than 6 months, in which a high proportion of cattle eliminated the parasite (4). It has previously been reported (14) that calves naturally infected with *B. bigemina* and isolated from infected ticks would eliminate the parasite more rapidly than would those infected with *B. bovis*. *B. bigemina* parasites were no longer detected in blood smears after 2 months of infection (14). The results obtained in the *B. bigemina* experimental infection study described here agree with those findings. However, subinoculation studies carried out 4 years after primary infection showed that a few animals were still infected (14). In a similar experiment, it was reported that latently infected cattle were found to be positive by subinoculation techniques 1 or 2 years after being freed of infected ticks (8).

Failure of animals 59 and 175 to seroconvert and, therefore, for infection to be detected by the complement fixation test and immunofluorescence assays could be due to the induction of a very weak antibody response by a very low level of parasitemia that is undetectable by light microscopy (animal 175), or the antibody response was against a different antigenic strain (animal 59), as has been reported elsewhere (5). The complement fixation test has been reported to have the lowest sensitivity, and detection of antibody to *B. bigemina* in cattle is reliable only for up to 4 months after a single infection (25). This would account for the negative complement fixation test result observed in all calves 7 months p.i. However, serology based on the immunofluo-

rescence assay technique has been reported to be accurate and sufficiently sensitive for the detection of *B. bigemina* reactors experimentally infected 18 to 24 months previously (21). Failure to detect antibody to *B. bigemina* in the presence of circulating parasites (as assessed by PCR) cannot be explained at this time. It could be argued, however, that the parasite populations detected in blood by the PCR method are immunologically different parasites which were present in and selected from the original population in the inoculum and, thus, induce antibody responses directed toward a variant population (3). This needs further experimentation.

In summary, in comparison with light microscopy, serology, in vitro culture isolation, and DNA probe analysis of unamplified DNA, the PCR-based assay proved to be more sensitive in detecting latently infected cattle over an 11-month period. The specificity and high analytical sensitivity of the test provide a valuable tool for performing large-scale epidemiologic studies in order to assess babesiosis in a geographic region. Once the prevalence of *B. bigemina* in a particular herd or region is known, adequate control measures can then be taken.

ACKNOWLEDGMENTS

This work was supported in part by USAID prime agreement no. DAN 4178-A-00-7056-00. Julio V. Figueroa was partially supported by INIFAP-SARH, Mexico.

We acknowledge the technical assistance provided by Karen K. McLaughlin and the secretarial assistance of Ellen Swanson.

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